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Small regulatory RNAs

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Chapter 1

Introduction

Introduction

Determining the function of genes and the complex regulatory interactions between them is an important objective within Life Science research. Before, the function of specific genes was primarily studied by inducing genetic mutations in targeted genes of interest. After the disruption of a specific gene, the to-be-translated functional protein will never be produced. These so called ‘knock-out’ studies allow the researcher to deduce the original function of the subjected gene, because the observed phenotypes are a consequence of the absence of a specific protein. A major drawback in classic knock-out studies using gene mutations is that it is relatively difficult to target specifically the genes of interest and it requires at least 1 or more generations before the model-organism has been designed and is ready for experiments. In addition, this type of experiments are rather crude because the gene is knocked out throughout the life cycle of the model-organisms which may have collateral effects not directly related to the knocked-out gene of interest. The RNAi (RNA Interference) technology has enriched and accelerated knock-out studies because it allows interfering at the RNA level [1]. This technique allows quantitative modification of RNA levels at any time and place of most organisms in a wild type background without the necessity to go through multiple generations of progeny of the model-organisms. By downregulating the mRNAs of the gene of interest, the functioning of the gene itself is abolished. This type of study allows straightforward gene expression manipulation and is also referred to as ‘knock-down’ study. RNAi-based research has led to the discovery of the microRNA (miRNA) pathway, which is involved in endogenous mechanisms of gene regulation. Multiple examples exist in which altered expression/activities of miRNAs are associated with pathologies.

The RNAi and miRNA pathway

RNAi exists as double stranded RNA of variable length which can enter an endogenous pathway known as the RNAi pathway. The RNAi pathway is responsible for the interference of messenger RNA (mRNA) translation based on sequence homology between the mRNA and the RNAi molecule. This thesis focuses on long double stranded RNA (dsRNA) of about 350 nucleotides (nt) in length, short interfering RNA (siRNA) of about 20-25 nt in length, and microRNAs (miRNAs) of about 20-25 nt in length.

In *Drosophila melanogaster*, RNAi is achieved by introducing an artificial dsRNA containing a sequence that is homologous to the gene of interest into cells, tissues or whole organisms to silence the translation of mRNAs of the targeted gene. By the action of the endogenous nuclease Dicer, this dsRNA is first cleaved into shorter fragments of about 20-25 nucleotides [2] and are referred to as the siRNAs. The siRNAs then split into single strand RNAs, of which one (the so called guide strand) is integrated into a protein complex known as the RNA-induced silencing complex (RISC) [3]. Although there are indications for a predominant determinant of which RNA strand will become the guide strand, other reports show that both are equally eligible for assembly into Argonaute, the catalytic component of the RISC complex [4, 5]. The RISC complex finds mRNAs that contain complementary sequences to the guide strand and actively cleaves the mRNA by the action of Argonaute which prevents translation. The siRNAs may also be included in the RNA-induced transcriptional silencing (RITS) complex. This complex can trigger posttranslational modification of histones resulting in a more permanent state of transcriptional silencing of the target gene [6]. This type of silencing is also referred to as epigenetics (see Figure 1 for an illustration of the different mechanisms) [7].

MicroRNAs (miRNAs) are produced from endogenous RNA encoding regions that regulate gene expression in a similar way as exogenous introduced RNAi molecules. The primary transcripts are processed within the nucleus to form a stem-loop structure called the pre-miRNA. The pre-miRNAs are cleaved by Dicer and therefore miRNA-induced events share much of the same downstream machinery as is controlled by Dicer and the RISC complex, but pre-miRNAs require some pre-modifications which is done by the microprocessor complex [8]. The miRNAs originate from noncoding regions and from intronic sequences [9]. They require limited homology to the target genes for being effective; only a stretch of 6-7 nt with 100% homology is sufficient and is denoted as the 'seed' region of the miRNA[10].

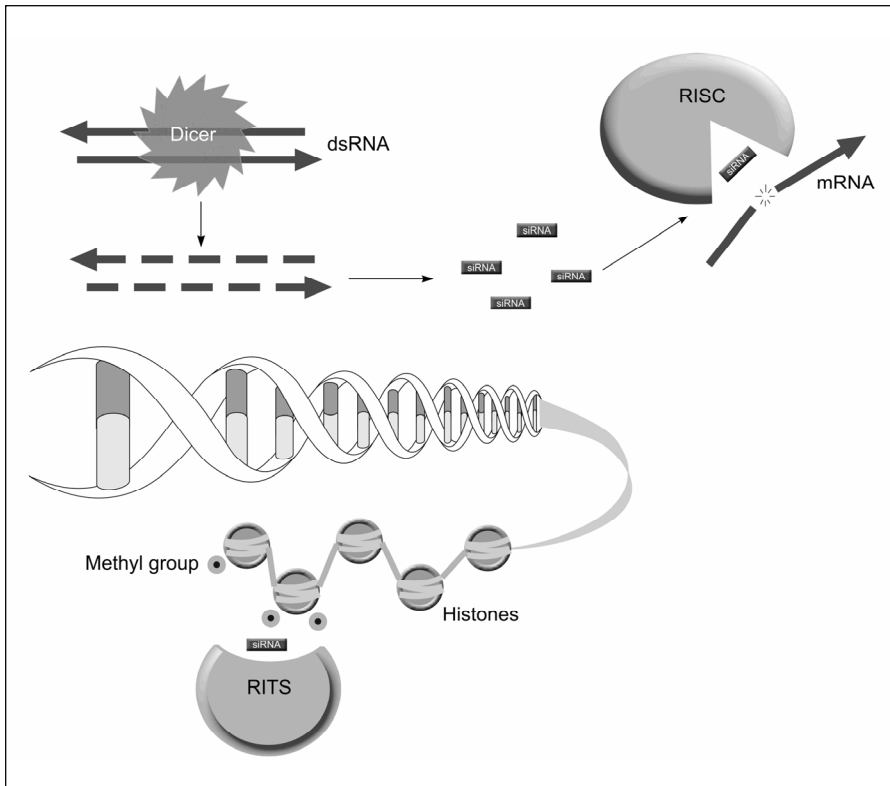


Figure 1 – Illustration of RNAi mechanisms. Dicer cuts the dsRNA into smaller siRNAs. The siRNAs is then either incorporated into RISC for RNA degradation or RITS for histone methylation.

Together, RNAs have been discovered as being very important in gene expression regulation, in a much more fine-tuned manner than the well-known gene promoter on-off actions [11]. RNAs are part of the regulatory system of living organisms (endogenously through miRNAs) and are effectively being exploited to influence the expression of the mRNA from targeted genes (exogenously through RNAi technology).

The specificity of knock-down studies

The biological results of the experiment in which the expression of specific genes are knocked down through RNAi can be difficult to interpret, because collateral effects can be present obscuring the relevant phenotype caused by the gene under study. These collateral effects are known as off-targets and are caused by (1) cellular responses due to the penetration of exogenous

RNA, and (2) through sequence homology of an RNAi construct with other genes than its intended target. Fortunately much has been discovered about the cellular responses, for example the interferon response which is triggered by double stranded RNA viruses [12]. By using strict design policies, and using proper transformation agents, these types of effects from (1) may be minimised or prevented altogether. However, off-targets caused by sequence homology (2) are still a challenge today [13-21] which will be discussed in more detail next.

An RNAi molecule is not exclusively specific because mismatches can be tolerated [10, 22] and not the complete length of an RNAi molecule is required to bind to mRNAs to induce a measurable effect [17, 18]. Birmingham et al. and Jackson et al. did a detailed analysis in human cell-based models and found that near-perfect matches (for example 18 matches throughout the whole siRNA) are sufficient to induce a change in protein expression [13, 17]. They, as well as others, also found that imperfect matches, apart from the 'seed' region of 6-nt at the 3' UTR (see further below) may contribute to off-targeting. Ma et al., Kulkarni et al. and Moffat et al. have shown that in *Drosophila*, many dsRNAs show off-target effects due to imperfect matches, as well as so called CAN repeats (N indicates any base) in low complex sequences within the dsRNA [19, 21]. Together their results reveal the strong evidence for false-positive rates in RNA interference (RNAi) screens using long dsRNAs.

It is also known that RNAi may target regions other than mature mRNAs, which greatly expands the possible unwanted targets to which RNAi may act upon [23-27]. The most relevant facts were found by Matzke et al., Boshier et al and Robb et al. who showed that RNAi can act within the nucleus, therefore other RNA molecules than mature mRNA can be influenced such as pre-mRNA containing intronic sequences.

Although still a challenge, bioinformatics may be of great help in analysing the genome and determining the specificity of RNAi molecules. As this thesis will show, this approach can help preventing many of the off-target effects which are caused by sequence similarities to non-targeted sequences.

Challenges for bioinformatics tools

Computers are a necessity during the design of RNAi constructs due to the large genomic data which need to be processed. Tools are available that

facilitate the search for siRNA candidates which have certain characteristics proven to show the best knock-down efficiency for the gene of interest (see table 1 for a comparison). This is done by gathering all known characteristics of identified highly potential siRNAs such as CG content and thermodynamic properties, and extrapolating this information onto the genome to find other highly active siRNAs [28]. Other tools are available that analyse siRNAs to find sequence similarities throughout the genome that might induce off-target effects [29]. Using the output of this off-target analysis, the most specific siRNA can be chosen amongst the many that are predicted to be potentially active.

Together, there are tools available that all have their own strength and weaknesses (see table 1 for a comparison). Unfortunately this has also led to misunderstandings. For example, BLAST (Basic Local Alignment Tool) is a very popular tool to perform all kinds of alignments. It is readily available through an on-line interface and is extremely fast in generating alignment reports. Because finding off-targets for a particular siRNA involves reviewing possible alignments against the genome, BLAST was also quickly adopted for this kind of work. However, the strength of BLAST lies particularly in large-to-large sequence alignments and is less useful for alignment studies of short sequences such as most siRNAs. BLAST uses a so called 'word size' that dictates the minimum contiguous sequence homology before any positive hit may be found. The on-line version of BLAST has a minimum word-size parameter of 7, which is necessary for the algorithm to perform within a timely fashion and allowing on-line data management. This minimum word-size of BLAST becomes a problem if a 21-nt sequence has for example 3 mismatches when aligned to a potential off-target sequence on the genome. These numbers of mismatches within a sequence can still provoke a potent RNAi response towards a complementary mRNA [19, 20, 22]. This complementary mRNA can be considered as an off-target effect. In case 3 mismatches are evenly spread across a 21-nt sequence, BLAST would require a minimum word size of $21/(3+1)=5.25$ to find this particular off-target. In other words, BLAST will miss this potential off-target with a word size of 7. This possibility is illustrated in Figure 2.

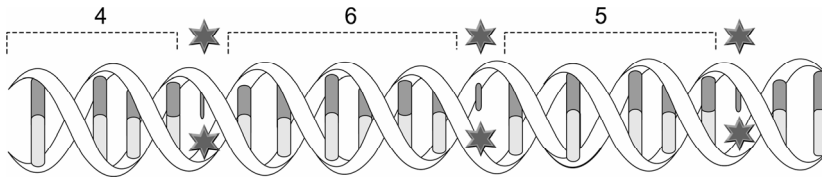


Figure 2 - Schematic representation of 2 single stranded RNAs that complementary bind with the exception of 3 positions (mismatches). The stars represent a mismatch due to incompatible nucleotides. This figure demonstrates in this example that the largest contiguous stretch of homology to be found is 6. The online version of BLAST however, requires at least 7 contiguous matches for a positive match. Blast will not identify this off-target, indicating that BLAST is not suitable for accurate off-target analysis.

There are tools that have smaller word sizes or have other methods for a more accurate alignment [28, 29]. For example there are tools available that are based on the Smith-Waterman algorithm which offers highly detailed alignment results [30]. However due to the long processing time, these types of algorithm are mostly used off-line with specialized computer systems [31-33]. Although it is possible to perform genome-wide analyses using these off-line systems, they take longer to complete than what is suitable for large scale and high-throughput siRNA analysis. Moreover the complexity of setting up these systems and lack of on-line availability makes them unreachable for non-bioinformaticians who do have the need for genome-wide off-target analysis.

Other tools are available that have different, more efficient algorithms that offer high-speed analysis without compromising sensitivity [28, 29]. These tools gain much of their acceleration by reducing the genome to non-redundant sequences that only include mature RNA (mRNA) sequences. This reduces the amount of nucleotides, which needs to be scanned, to a great extent. This in turn results in a significant increase of the performance and in creating the possibilities for widely adoptable online tools or high-throughput analysis. The general idea behind this approach was that RNAi is believed to be exclusively active within the cytoplasm and therefore will act upon mRNA sequences only [34]. Based on this assumption, it would indeed be sufficient to search only within mRNA sequences. However, research papers have reported evidence about RNAi being active within the nucleus, indicating that the previous assumption is not correct [23-27].

Chapter 2 of this thesis presents a novel tool called RNAi-Select to reduce off-target effects by performing highly sensitive sequence comparisons using a novel algorithm that is more comprehensive and appropriate than existing tools. By generating a comprehensive sequence alignment report, the user is able to choose a particular RNAi molecule with high specificity and by using this selected RNAi molecule off-targets can be prevented as much as possible.

	RNAi-Select	BLAST	dsCheck / siDirect
Freely on-line available application	Yes	Yes	Yes
Speed	Fast	Fast	Fast
Accurate short sequence alignment	Yes	No	Yes
Finds 1 (G:U or other) mismatches	Yes	Yes	Yes
Finds 2 (G:U or other) mismatches	Yes	No	Yes
Finds 3+ (G:U or other) mismatches	Yes	No	No
Finds exon or UTR based siRNA off-targets	Yes	Some	Yes
Finds whole genome siRNA off-targets, including introns	Yes	Some	No
Finds seed based miRNA off-targets	Yes	No	No
Designs specific shRNAs to knockdown <i>Drosophila</i> genes ¹	Yes	No	No
Average validated off-targets found per dsRNA ²	13	0	2
Identification of non-overlapping dsRNAs with no shared off-targets	Yes	No	No

Table 1 – Comparison and features of RNAi-Select and two other on-line available tools

A double controlled strategy to circumvent off-targets

In many published manuscripts, two independent RNAi constructs have been used in separate experiments to downregulate the gene of interest [35-37], because previously it was demonstrated that this strategy allows to filter for off-target effects [20, 38]. The assumption is that when two non-overlapping RNAi constructs induce the same phenotype, this phenotype is an on-target effect and most likely not an off-target effect. This is reasonable because any phenotypical observation that occur in both dsRNA experiments is expected to be the result of downregulation of the intended target and not from any off-targets. Because it is also reasonable to assume

that each dsRNA has its own unique set of possible off-targets. In other words, any induced effect that occurs only after the use of one dsRNA both not in the other is attributed to off-target effects of that particular dsRNA and should be disregarded. Again, this postulation is based on the assumption that each dsRNA has a unique off-target profile, however this has never been tested experimentally. One major flaw in this assumption is that an off-targeted mRNA does not exist of a short 21-nt fragment, but is in itself most of the time a sequence of more than 400 nt with a large active surface having many possibilities of having sequence similarities to siRNAs. Thus even though different non-overlapping dsRNAs may have a completely different sequence composition, in theory their derived siRNAs can still bind to the same off-target mRNA albeit at different sites. Figure 3 presents an illustration of this possible event.

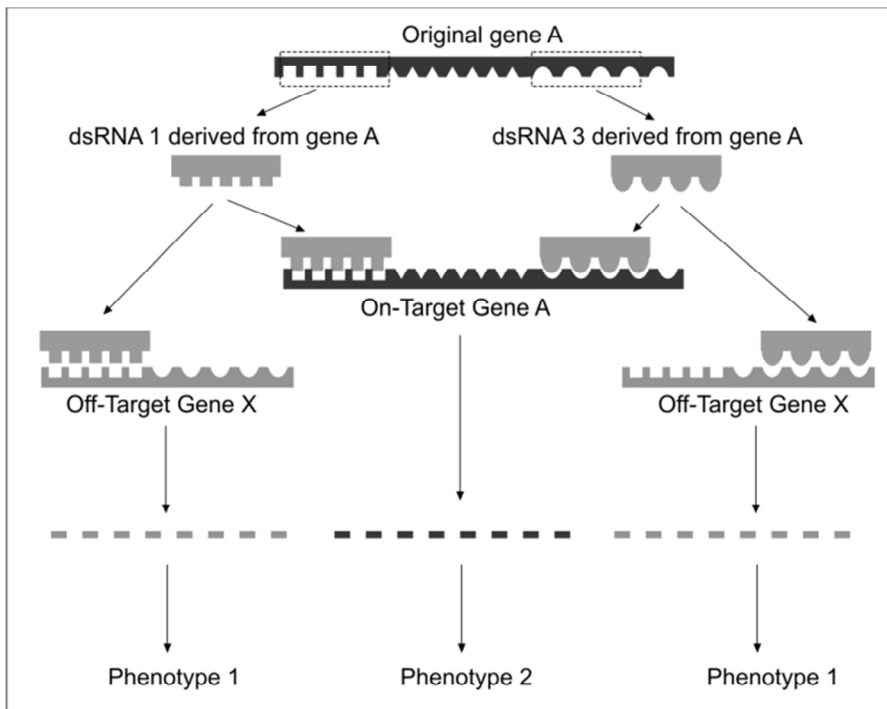


Figure 3 – Schematic representation of 2 non-overlapping dsRNAs that have sequence similarity to the same off-target.

Chapter 3 presents evidence that non-overlapping sequences derived from the same gene have an almost 100% change of sharing identical potential off-targets. We present a proper approach to select multiple dsRNA

molecules derived from the gene to be targeted that do not share off-targets. These “clean” RNAi constructs can be used to investigate the consequences of downregulation of a specific gene in the absence of predicted off-target effects. Any phenotypical observations that occur after using both RNAi molecules in separate experiments are then expected to originate solely from its intended target and not from any off-targets for which each RNAi molecule has its own unique set.

Performing a controlled RNAi experiment to model the human disease PANK in *Drosophila melanogaster*

In chapter 4, the new tools as described in the previous chapters were used to design “clean” RNAi constructs used in experiments to study the consequences of downregulation of pantothenate kinase, a gene associated with the human neurodegenerative disease PKAN (Pantothenate kinase-associated neurodegeneration). The disease is characterized by an early onset during childhood and the symptoms are: brain abnormalities, locomotion dysfunction and early death [39]. PKAN is caused by mutations in the pantothenate kinase 2 gene (PANK2), which is the first enzyme in the pathway responsible for the conversion and synthesis of Coenzyme A (CoA) from its precursor vitamin B12 [40]. CoA is an essential metabolic cofactor for many biochemical reactions such as the citric acid cycle and fatty acid oxidation.

A PKAN mouse model was created in which the mouse PANK2 gene was disrupted [41]. Unfortunately this mouse model lacks the neurodegenerative characteristics of PKAN, most likely because mouse PANK2 is in contrast to human PANK2 not localized in mitochondria [42, 43]. These data indicate that using the mouse model is not appropriate to understand the underlying mechanisms of PKAN. However, in *Drosophila melanogaster*, mutations in *fumble* (the *Drosophila* PANK2 orthologue, further referred to as dPANK/fbl) induce neurodegeneration, locomotion dysfunction and an early death [44]. These data indicate that the *Drosophila* model is appropriate to perform PKAN-related research. A cell line derived from a primary culture of late stage (20-24 hours old) *Drosophila* embryos (S2-cells) was used to create a cell model for PKAN with the use of RNAi. Using the newly developed algorithm as described in chapter 2, a dsRNA construct has been designed devoid of most of the predicted potential off-targets. Western blot analysis revealed a highly efficient knock-down of

dPANK/fbl. The S2 cell model in combination with RNAi technology provided us with a tool to analyse biochemical abnormalities as a result of impaired dPANK/fbl function. In addition different compounds were tested for their rescuing potential towards the dPANK/fbl-depleted phenotype. Chapter 4 describes the observations and the effects of different compounds based on this S2 model. Findings in the S2 cells were further investigated in the *Drosophila* mutants and appeared to be highly similar.

miRNAs and identifying their targets using RNAi-Select in Hodgkin Lymphoma

In chapter 5, a new bioinformatics tool related to the previous tools was developed to understand the human disease Hodgkin Lymphoma. Hodgkin Lymphoma is a cancerous disease, originating from irrepressible dividing white blood cells (lymphocytes) and characterized by an aberrant miRNA profile [45].

RNA regulation is a natural phenomenon and not restricted to exogenously delivered siRNAs. In fact, endogenous miRNAs are vital during cellular development [46, 47]. In addition, miRNA malfunction has been associated with various pathological conditions including cancer [48-51] due to regulatory imbalances. Studying miRNAs and their targets is a key factor towards understanding the pathology of many cancerous diseases. Unfortunately proper tools allowing genome-wide identification of miRNA targets are lacking. In part because of technical difficulties to detect miRNA changes in biological material, but also due to the nature of miRNAs which allow a much shorter length of sequence homology to be active as compared to siRNAs. A sequence as short as 6-7 nucleotides (the so-called seed region) has proven to induce regulatory effects [52], this is a remarkable finding because any random 6 nucleotide sequence is expected to be found tens of thousands of times in a genome with the size of *Drosophila* (see formula 1 in supplementary file Chapter 3). Obviously, miRNAs or their possible targets must have some other properties that allow for a more specific regulatory function, like for example spatial or temporal expression differences. Also the position of the homologous site on the target mRNA and enriched regions that contain multiple copies of a certain seed region may play a role in determining the real targets. A bio-informatics challenge is to find/predict the natural targets of miRNAs, because based on sequence

homology (or similarity) there are numerous candidates and numerous false positive hits.

Chapter 5 describes an in-depth analysis to find miRNA targets in human cells. By combining bioinformatics and ‘wet’ experiments, many genes were identified in which the regulation show a strong correlation with the expression of the miRNAs *miR-16*, *miR-21*, *miR-24* and *miR-155* that are involved in Hodgkin Lymphoma [45]. Research in this field is important as regulatory malfunction is characteristic for cancerous diseases in general and miRNAs are being discovered for playing a central role in them [53].

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